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Validation of the sperm mobility assay in boars and stallions

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Abstract

The sperm mobility assay used in the present study measures the rate of sperm penetration in a biologically inert cell-separation solution (Accudenz). When a sample of sperm is overlaid in a cuvette containing Accudenz, sperm penetrate the solution and absorbance of the sample can be measured with a spectrophotometer. This assay has been successfully used to select chicken and turkey semen donors. We validated this assay for semen from boars and stallions. Absorbance was measured after overlaying fresh semen from each species in prefilled cuvettes for 1, 5, 10, 15, 20, and 40 min. There were no significant differences when sperm were incubated in prewarmed cuvettes at 37, 39, or 41 °C. However, a minimum concentration of 5×10^7 viable sperm/mL was needed to evaluate the rate of sperm penetration in boars. Absorbance was half-maximal at 5.4 and 14.1 min for boar and stallion sperm, respectively. Frequency analysis suggested a normal distribution of mobility values for boar sperm. There were positive correlations between mobility values and several computer-aided sperm analysis (CASA) parameters. In addition, there was medium repeatability for multiple ejaculates from single males. We concluded that the mobility assay can be used for mammalian sperm and there seemed to be phenotypic variation among boars in mobility estimates.

Keywords: Mobility; Sperm; CASA; Boars; Stallions

1. Introduction

Most laboratories and breeding farms use a number of different tests in an attempt to evaluate the fertility of a sperm sample. For instance, sperm motility and morphology, sperm membrane integrity, number of sperm per ejaculate, and ejaculate volume are routinely assessed in AI centers. However, there is no good evidence that any of these characteristics are significantly correlated with fertility in stallions [1,2] or in boars [3–6]. Although in vitro fertilization of homologous oocytes was a superior method to assess fertility, it is time-consuming and technically difficult for field application [3].

Computer-aided sperm analysis (CASA) has been used to evaluate sperm motility parameters in several species including boars [7]. However, the ability of CASA to predict fertility in individual males is questionable.

The development of a simple, inexpensive in vitro test of male fertility remains an important goal for the horse and swine industries. The rate of sperm penetration in an Accudenz solution (net movement of sperm) is a quantitative method to evaluate motility of avian sperm [8]. Accudenz is a non-ionic, biologically inert, cell-separation solution. A known concentration of sperm is overlaid on a cuvette that was previously filled with Accudenz. After a 5-min incubation, the absorbance of the Accudenz solution is measured in a spectrophotometer and the absorbance

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is proportional to the number of sperm that have entered the Accudenz layer [8]. In other words, the spectrophotometer measures light transmission that is indicative of the ability of the sperm sample to swim down the cuvette; thus, the relative mobility of the ejaculate is obtained. Froman et al. [9] reported that fecundity of artificially inseminated hens (*Gallus domesticus*) could be increased when sperm penetration of Accudenz was used as a selection criterion for semen donors. Similarly, semen donor selection by in vitro sperm mobility increased fertility in the turkey hen [10,11]. The cellular basis underlying the phenotypic variation of sperm mobility in poultry populations has not been determined. However, high correlations between sperm oxygen consumption and sperm mobility have been reported ($r = 0.83$; [12]). Despite the remarkable advantage of this method to evaluate fertility in roosters, there are apparently no reports regarding the use of this procedure with semen from boars or stallions. Therefore, the objectives of the present experiments were to: (1) validate the mobility assay in boars and stallions, (2) assess the phenotypic variation of mobility values among boars, (3) evaluate the repeatability of the mobility values in boars, and (4) determine the correlation of the mobility values with CASA measurements in boars.

2. Materials and methods

2.1. Mobility assay general procedure

Mobility was quantified using a technique similar to that described by Froman and McLean [8]. In brief, a 30% (w/v) stock solution (pH 7.4) of Accudenz (Accurate Chemical & Scientific Corp., Westbury, NY, USA) was prepared with 3 mM KCl (Fisher Scientific, Pittsburgh, PA, USA) and 5 mM TES (*N*-tris[hydroxymethyl]methy-2 aminoethanesulfonic acid; Sigma–Aldrich Co., St. Louis, MO, USA). In addition, a mobility buffer (pH 7.4) containing 111 mM NaCl, 25 mM glucose, and 4 mM $CaCl_2$ (Fisher Scientific) in 50 mM TES was diluted in distilled water to an osmolality of 290 mM/kg. Finally, a 6% (w/v) solution was obtained by diluting (1:5) of the stock solution with the mobility buffer. A 3-mL volume of the 6% solution was aliquoted into several polystyrene cuvettes until it was used to evaluate mobility of various sperm samples.

2.2. Animals

Boars were obtained from Texas Tech University (TTU; $n = 4$; 17–48 months of age; Hampshire x Duroc meat type sires; with good fertility), and from USMARC (Clay Center, NE; $n = 47$, 15–17 months of age; a four-breed composite of maternal Landrace, Yorkshire, Duroc and terminal, lean line Landrace). Only one-half of the USMARC boars were used for breeding; fertility of these boars was good. Stallions ($n = 8$; Quarter horses, with known good fertility) were owned either by the Texas Tech Ranch Horse Center or by producers. Fresh ejaculates were collected by experienced operators using the gloved-hand method (boars) or a Missouri-style artificial vagina (stallions). Sperm concentration from each animal was tested with a precalibrated densimeter (Animal Reproduction Systems, Chino, CA, USA) that was regularly used for this purpose. Additionally, fluorometric determination of sperm viability was documented as previously described [13].

Fresh semen (boars from TTU) or stored ejaculated semen (15.9 ± 0.1 °C for 24 h; boars from USMARC) was diluted to 1×10^8 viable sperm/mL with Androhep® (Minitube of America, Verona, WI, USA) in Experiments 1, 2, and 3, and with Beltsville Thawing Solution (Minitube of America) in Experiment 4. Fresh semen from each stallion was diluted to 1×10^8 viable sperm/mL with E-Z Mixin® (Animal Reproduction Systems). Procedures used to obtain semen were approved by the TTU and the USDA MARC Animal Care and Use Committee, and were part of the Standard Operation Procedures for both institutions.

2.3. Experiment 1

Initially, the ejaculates of four boars and four stallions were evaluated in replicates for their penetration through Accudenz. A known concentration of fresh viable sperm cells (1×10^8) was carefully overlaid (300 μL) on a cuvette previously filled with Accudenz and prewarmed to one of three temperatures (37, 39, or 41 °C). Sperm from a fresh ejaculate that was previously heated at 60 °C for 10 min was used as a negative control. Sperm from a fresh ejaculate that was previously mixed with Accudenz was used as a positive control. Using a spectrophotometer, absorbance (550 nm) was recorded at 1, 5, 10, 15, 20, and 40 min. Split-plot analyses of variance were used to determine the effect of treatment (temperatures) over time on percent absorbance. The PROC MIXED procedure of SAS [14] for repeated measures was used. Animals within treatment were used as the error term to test treatment effects, and animals within treatment by time were used as the error term to test time and the interaction of time with treatment.

2.4. Experiment 2

Ejaculates were collected from four boars and further diluted with Androhep to 1×10^8, 5×10^7, and 1×10^7 viable sperm/mL. Additionally, the ejaculate from four stallions was collected and used either without dilution (undiluted) or further diluted with E-Z Mixin to 1×10^8 and 5×10^7 viable sperm/mL. Sperm were carefully overlaid (300 μL) on a cuvette previously filled with Accudenz and prewarmed to 37 °C. Percent absorbance (550 nm) was recorded at 1, 5, 10, 15, 20, and 40 min.

Split-plot analyses of variance were used to determine the effect of treatment over time on absorbance. The PROC MIXED procedure of SAS [14] for repeated measures was used. Animals within treatment were used as the error term to test treatment (i.e. dilutions) effects, and animals within treatment by time were used as the error term to test time and the interaction of time with treatment. If the time by treatment interaction was significant, the following approach was used. The relationship between absorbance and time was evaluated using a non-linear model (absorbance = $G_1/[1 + K_m/\text{time}] - G_2$). The time at which the absorbance was half-maximal (K_m) was characterized for each boar, stallion, replicate, and dilution. To evaluate differences between sperm concentrations, the different K_m's were analyzed as a completely randomized design. Duncan's multiple range test was used to compare treatment means.

2.5. Experiment 3

Stored semen (24 h) from 24 boars obtained from USMARC was manipulated as described above. Upon arrival at TTU, sperm was diluted to 5×10^7 viable sperm/mL and maintained at 37 °C. A 300-μL volume of sperm suspension was overlaid in a cuvette containing 6% Accudenz. After incubating for 5 min at 37 °C, the cuvette was removed from the water bath, placed within the spectrophotometer, and the absorbance at 550 nm was recorded. This process was repeated for all sperm samples. A frequency analysis of the absorbance obtained from individual animals was performed. A Shapiro-Wilk test was used to test the hypothesis that the observed frequencies approximated a normal distribution (PROC UNIVARIATE procedure of SAS [14]).

2.6. Experiment 4

Weekly collection of semen from boars was initiated 5 week before the start of this experiment. Stored semen (24 h) from 23 boars (USMARC) was obtained once weekly for 3 consecutive weeks. Upon arrival at TTU, sperm was diluted to 5×10^7 viable sperm/mL and maintained at 37 °C. A 300-μL volume of sperm suspension was overlaid in a cuvette containing 6% Accudenz. After incubating for 5 min at 37 °C, the cuvette was removed from the water bath, placed within the spectrophotometer, and the absorbance at 550 nm was recorded. Additionally, Computer-Aided Sperm Analysis (CASA; Minitube SpermVision system; Minitube of America) was performed in one location (USMARC), in fresh (day of collection) and stored (24 h) sperm samples as previously described [15]. Parameters measured by CASA included the following: curvilinear velocity (VCL); average path velocity (VAP); straight line velocity (VSL); beat cross frequency (BCF); linearity (LIN); and progressive motility (PGM). Definitions and validation of these parameters have been reported elsewhere [15]. Analysis of covariance was performed to estimate the coefficient of repeatability (CR) in all parameters described above. The PROC MIXED procedure of SAS [14] was used to obtain the variance component for differences among boars (S_A^2), and the variance component for differences within boars (S_E^2). The CR was defined by using the following formula: $\text{CR} = S_A^2/[(S_A^2 + S_E^2)/n]$, where n is the number of repeated measurements in each boar ($n = 3$). Pearson's correlations were performed between the sperm mobility values and the CASA parameters (PROC CORR procedure of SAS [14]).

3. Results

3.1. Experiment 1

When fresh boar or stallion semen was overlaid in prewarmed cuvettes, sperm cells rapidly entered the Accudenz solution and, as a consequence, a significant increase in absorbance was attained over time. However, no significant treatment or treatment by time interaction was observed (Figs. 1 and 2). Sperm rendered immotile by heating at 60 °C did not penetrate the Accudenz layer. Positive control samples had constant absorbance over time.

3.2. Experiment 2

When fresh semen from boars was diluted to 1×10^8, 5×10^7, and 1×10^7 viable sperm/mL, there was a treatment by time interaction ($P < 0.01$; Fig. 3). In addition, the time at which the absorbance was half-maximal (K_m) differed among dilutions ($6.9^a \pm 1.2$,

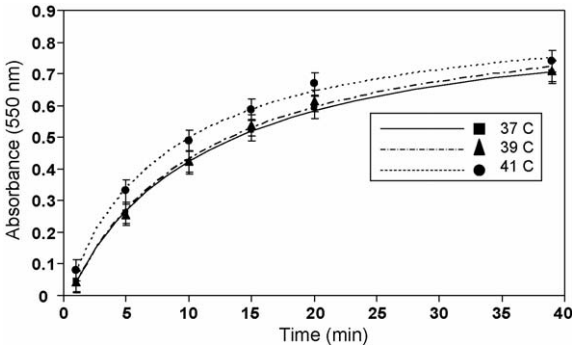

Fig. 1. Least square non-linear regression (line) and means (symbols) for the relationship between absorbance (550 nm) over time for boar sperm migrating in an Accudenz solution. Symbol (±S.E.M.) represents an average of four ejaculates measured in duplicate over time.

$5.2^{a} \pm 1.1$, and $1.7^{b} \pm 1.1$ min for 1×10^8, 5×10^7 and 1×10^7 viable sperm/mL, respectively; means ± S.E.M. with different superscripts differ, $P < 0.05$).

When fresh semen from stallions was used as undiluted or diluted to 1×10^8 and 5×10^7 viable sperm/mL, there was a treatment by time interaction ($P < 0.01$; Fig. 4). However, the time at which the absorbance was half-maximal (K_m) was not significantly affected by sperm concentration (14.1 min, average for undiluted, 1×10^8 and 5×10^7 viable sperm/mL).

3.3. Experiment 3

A frequency analysis for absorbance of the ejaculate was determined for 24 boars. Sperm (5×10^7 viable sperm/mL) were overlaid in a cuvette previously warmed to 37 °C for 5 min. Data were slightly negative

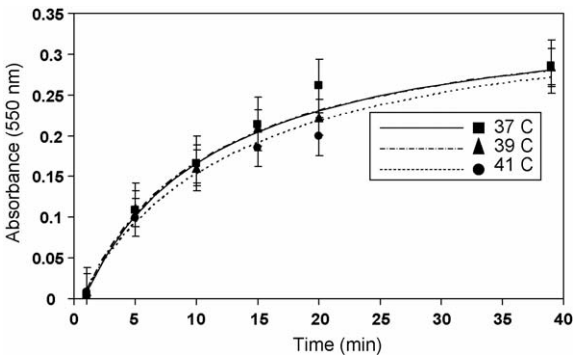

Fig. 2. Least square non-linear regression (line) and means (symbols) for the relationship between absorbance (550 nm) over time for stallion sperm migrating in an Accudenz solution. Symbol (±S.E.M.) represents an average of four ejaculates measured in duplicate over time.

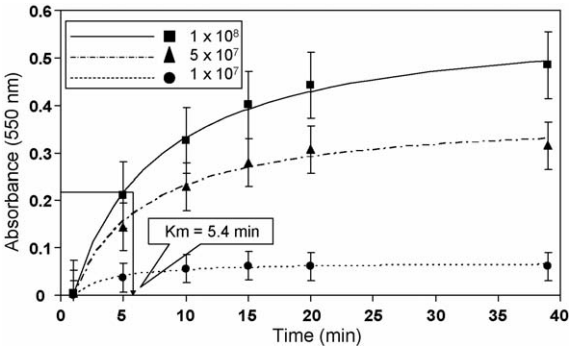

Fig. 3. Least square non-linear regression (line) and means (symbols) for the relationship between absorbance (550 nm) over time for boar sperm migrating in an Accudenz solution. Symbol (±S.E.M.) represents an average of four ejaculates measured in duplicate over time. The K_m represents the average time at which the absorbance was half-maximal for the two highest sperm concentrations.

skewed (skewness = −0.17) and somewhat platykurtic (kurtosis = −0.46). Nonetheless, using a Shapiro-Wilk test, the hypothesis that the observed frequencies approximated a normal distribution was not rejected ($P = 0.55$; Fig. 5). The predicted normal distribution (solid line in Fig. 5) was obtained using estimates of $\mu = 0.567$ and $\sigma = 0.155$.

3.4. Experiment 4

The coefficient of repeatability ($0 < CR < 1$) for mobility values was 0.56. The CR for various CASA parameters, when measurements were made in stored semen, were 0.00, 0.19, 0.52, 0.22, 0.21, and 0.32 (for PGM, BCF, LIN, VAP, VCL, and VSL, respectively). When CASA measurements were made in fresh semen,

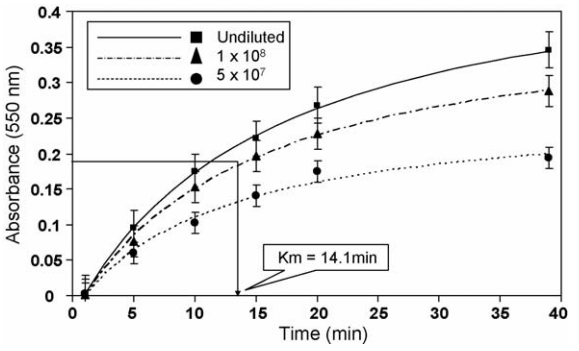

Fig. 4. Least square non-linear regression (line) and means (symbols) for the relationship between absorbance (550 nm) over time for stallion sperm migrating in an Accudenz solution. Symbol (±S.E.M.) represents an average of four ejaculates measured in duplicate over time. The K_m represents the average time at which the absorbance was half-maximal for all sperm concentrations.

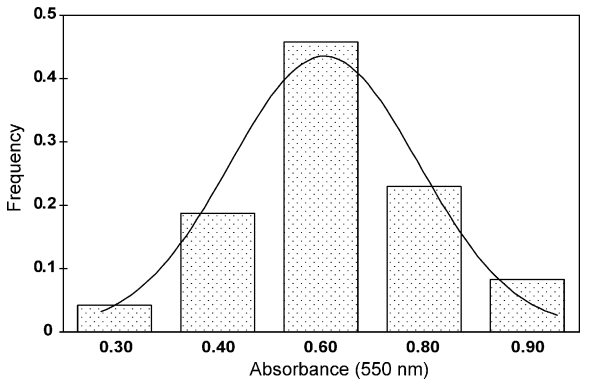

Fig. 5. Frequency (bars) and normal distribution (line) of absorbance from sperm of 24 boars migrating in an Accudenz solution. Sperm cells were stored for 24 h before absorbance was measured. Bars represent the frequency of sperm mobility in samples that were diluted to 5×10^7 viable sperm/mL, and overlaid in prewarmed cuvettes (37 °C) for 5 min, before absorbance (550 nm) was recorded. The solid line depicts the normal curve as determined by the normal distribution density function.

Table 1
Pearson correlations coefficients between mobility values (measured in an Accudenz solution) and several computer-aided sperm analysis (CASA) parameters performed in fresh samples of boar sperm

	PGM[a]	BCF	LIN	VAP	VCL	VSL
Mobility	0.24*	0.38**	0.15	0.28*	0.27*	0.31*
PGM		0.54**	0.14	0.60**	0.57**	0.56**
BCF			0.56**	0.72**	0.51**	0.82**
LIN				0.30*	−0.20	0.60**
VAP					0.86**	0.93**
VCL						0.65**

[a] PGM: progressive motility; BCF: beat cross frequency; LIN: linearity; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity.
* $P < 0.05$.
** $P < 0.005$.

the CR was reduced for most parameters (0.00, 0.06, 0.51, 0.00, 0.00, and 0.00 for PGM, BCF, LIN, VAP, VCL, and VSL, respectively). There were significant positive correlations between sperm mobility, and PGM, BCF, VAP, VCL, and VSL for CASA measurements of fresh semen (Table 1). However, for stored semen, there were no significant correlations between CASA parameters and mobility values.

4. Discussion

The first objective of the present research was to evaluate the optimal temperature of the mobility assay. In chickens, the mobility assay is carried out at 41 °C [8]. However, unlike most mammalian species, male chickens have internal testes and spermatogenesis occurs at the normal avian temperature of 41 °C. We hypothesized that the temperature required for mammalian sperm cells to enter the Accudenz solution may be lower than the one used in chickens. After natural mating or artificial insemination, sperm cells are exposed to the normal internal body temperature of the female reproductive tract. There were no significant differences in absorbance over time, when cuvettes were maintained at 37, 39, and 41 °C suggesting that temperature of the cuvette did not affect the ability of sperm cells to penetrate the Accudenz layer (Figs. 1 and 2). Therefore, we adopted a temperature of 37 °C for the next experiments.

The second objective was to characterize the minimum concentrations of sperm cells needed to enter the Accudenz solution. Normal average concentrations of undiluted sperm for boars and stallions are 1–3×10^8 sperm cells/mL [16]. In roosters, sperm concentration normally average 35×10^8 sperm/mL; 5×10^8 sperm/mL are used in the mobility assay [8]. We hypothesized that fewer boar and stallion sperm than chicken sperm would be needed to enter the Accudenz solution. In fact, there was a significant time by dilution interaction for both boar and stallion sperm. In boars, the time at which the absorbance was half-maximal (K_m), did not differ between 1×10^8 and 5×10^7 viable sperm/mL. When data from the two highest dilutions was pooled, a K_m of 5.4 was obtained (Fig. 3). Therefore, we adopted a concentration of 5×10^7 viable sperm/mL and a 5-min incubation before placing the cuvette within the spectrophotometer. In the stallion, there were no significant differences among K_m's when undiluted, 1×10^8 and 5×10^7 viable sperm/mL were used. Therefore, the minimum sperm concentrations to be used in stallions cannot be characterized from this experiment. When pooled data from all concentrations was analyzed, the time at which the absorbance was half-maximal was 2.6 times greater in stallions compared with boars (K_m 14.1 min versus 5.4 min for stallions and boars, respectively). To the best of our knowledge, this is the first report on the use of the mobility assay in mammalian species. Our data do not explain the differential K_m obtained in boars and stallions. However, a significant correlation between mobility, sperm oxygen consumption, and sperm ATP content has been reported in chickens [12,17]. Since ATP content in boar sperm is almost twice as much as that in stallion sperm [18], perhaps differences in ATP content, sperm oxygen consumption, or both, were responsible for the difference between species.

The third objective of this research was to assess the phenotypic variation of the mobility values in stored semen from boars. Unfortunately, the number of stallions available for this experiment was limited and phenotypic variation of the mobility values in horses was not evaluated. As indicated by ranked absorbance (Fig. 5), there were considerable differences in sperm mobility among ejaculates of different males. Additionally, there was a normal distribution of the rate at which the sperm penetrated the Accudenz solution. Similarly, there was a normal distribution of sperm mobility in domestic fowl [17], suggesting that the sperm mobility is a quantitative trait in chickens. In addition, a strong positive correlation between sperm mobility and fertility was obtained in chickens and turkeys [10,19]. Unfortunately, the limited use of USMARC boars for breeding did not allow us to evaluate the association between mobility and fertility.

The fourth objective of the present research was to evaluate the repeatability of the mobility values and the correlation between mobility and various CASA parameters. The relatively medium repeatability for mobility (CR = 0.56) was severely influenced by high variability within weeks for a few boars. In fact, if data from five highly variable boars were removed, the coefficient of repeatability significantly increased to 0.76. We inferred that mobility should be measured in duplicates to account for some of the variation associated with this procedure. Nevertheless, repeatability for mobility was higher than any of the CASA parameters evaluated in fresh or stored semen. To the best of our knowledge, this is the first report on the repeatability of mobility and CASA parameters in boars. When CASA measurements were performed in fresh semen, correlations between PGM, BCF, VAP, VCL, VSL, and sperm mobility were positive and similar to those previously reported in turkeys [20]. The significant positive correlation between mobility and the velocity over a total distance (VCL) or the velocity over a calculated smoothed path (VAP) suggested that sperm cells with higher mobility, had the ability to move faster than sperm cells with low mobility. Moreover, the positive association between mobility and the velocity calculated using a straight-line distance between the beginning and end of the sperm track (VSL) suggested that sperm with high mobility not only had the ability to move faster, but also to achieve that velocity with a straighter track. Finally linear progression (PGM and BCF) were also positively associated with mobility. It is not clear why correlations between CASA parameters and mobility values were not significant when CASA measurements were made in stored semen.

In conclusion, we inferred that the sperm mobility assay can be used for mammalian sperm and that a normal distribution of this trait was apparent in boars, and has potential for other species (e.g. cattle). Additional data are needed to evaluate the ability of this assay to predict fertility in boars and stallions.

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